

Regionally Reduced Brain Volume, Altered Serotonin Neurochemistry, and Abnormal Behavior in Mice Null for the Circadian Rhythm Output Gene *Magel2*

Rebecca E. Mercer,¹ Erin M. Kwolek,¹ Jocelyn M. Bischof,¹ Matthijs van Eede,² R. Mark Henkelman,^{2,3} and Rachel Wevrick^{1*}

¹Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada

²Hospital for Sick Children, Mouse Imaging Centre, Toronto, ON, Canada

³Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada

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Magel2 belongs to the MAGE/necdin family of proteins, which have roles in cell cycle, differentiation, and apoptosis. The *Magel2* gene is expressed in various brain regions, most notably the hypothalamus. Mice with a targeted deletion of *Magel2* display hypoactivity, blunted circadian rhythm, decreased fertility, and increased adiposity. The human ortholog, *MAGEL2*, is one of a set of paternally expressed, imprinted genes inactivated in most cases of Prader–Willi syndrome, a complex neurodevelopmental disorder. To explore the role of *Magel2*, brain morphology, brain neurochemistry, and behavior were measured in *Magel2*-null mice. Brain volume was reduced in specific regions, particularly in the parieto-temporal lobe of the cerebral cortex, the amygdala, the hippocampus, and the nucleus accumbens, as measured by quantitative magnetic resonance imaging. Abnormal neurochemistry was detected in brain samples from adult mice, consisting of decreased serotonin and 5-hydroxyindoleacetic acid in the cortex and the hypothalamus, and decreased dopamine in the hypothalamus. *Magel2*-null mice displayed relatively normal motor and learning abilities, but exhibited abnormal behavior in novel environments. This study lends support to the important role of the circadian rhythm output gene *Magel2* in brain structure and behavior. © 2009 Wiley-Liss, Inc.

Key words: Prader–Willi syndrome; anxiety; magnetic resonance imaging; circadian rhythm; imprinting

INTRODUCTION

Magel2 is a member of the Type II melanoma-associated antigen gene (MAGE) protein family, which share a protein–protein interaction domain called the MAGE homology/conserved domain [Chomez et al., 2001; Barker and Salehi, 2002]. Type II MAGE proteins include necdin, *MAGEL2*, *MAGED1/NRAGE*, *MAGEG1*, and *MAGEH1*, and are abundantly expressed in normal (non-tumor) somatic cells [Maruyama et al., 1991; Boccaccio et al., 1999; Lee et al., 2000; Salehi et al., 2000; Chibuk et al., 2001; Barker and Salehi, 2002]. In contrast, Type I MAGE genes are expressed only in cancer cells and in testis [De Plaen et al., 1994, 1999; De Backer et al.,

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Abbreviations: Acb, nucleus accumbens; Amg, amygdala; BST, bed nuclei of the stria terminalis; CBX, cerebellum; DA, dopamine; Dgh, dentate gyrus of the hippocampus; DOPAC, dihydroxyphenylacetic acid; DI, discrimination index; FDR, False Discovery Rate; HPLC, high performance liquid chromatography; HVA, homovanillic acid; HY, hypothalamus; LSX, lateral septal complex; MAGE, melanoma-associated antigen gene; *MAGEL2*, melanoma-associated antigen gene L2; MEA, medial amygdalar nucleus; MRI, magnetic resonance imaging; MS, medial septal nucleus; MY, medulla; NA, noradrenaline; PBS, phosphate-buffered saline; Ptc, parieto-temporal lobe of the cerebral cortex; PWS, Prader–Willi syndrome; SEM, standard error of the mean; TH, tyrosine hydroxylase; WT, wild-type control; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.

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*Correspondence to:

Rachel Wevrick, Department of Medical Genetics, University of Alberta, 8-16 Medical Sciences Building, Edmonton, Alberta, Canada T6G 2H7. E-mail: rachel.wevrick@ualberta.ca

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1995]. The MAGE homology domain of several Type II MAGE proteins (necdin, MAGE-G1, MAGED1) binds to multiple transmembrane receptors involved in intracellular signaling [Salehi et al., 2000; Tcherpakov et al., 2002; Kuwako et al., 2004]. *Magel2* is expressed in the developing murine central nervous system and in cell lines that model neuronal differentiation [Boccaccio et al., 1999; Lee et al., 2000]. *Magel2* expression is apparent in the hypothalamus, the control center in the brain for endocrine function, circadian rhythm, appetite, thirst, and thermoregulation [Moore et al., 2002], with maximal levels in the suprachiasmatic nucleus (a region of the anterior ventral hypothalamus) and the arcuate nucleus [Lee et al., 2000, 2003].

MAGEL2 and *NDN*, the gene that encodes the MAGE protein necdin, are among a small set of genes that are typically inactivated in Prader–Willi syndrome (PWS). PWS is a congenital disorder characterized by symptoms of varying severity among affected individuals: intellectual disability, hypotonia, short stature, childhood-onset hyperphagia often leading to obesity, excessive sleepiness, neuroendocrine abnormalities, and incomplete sexual development [Gunay-Aygun et al., 2001; Eiholzer and Whitman, 2004; Goldstone, 2004]. While physiological abnormalities in PWS point to a defect in the development or function of the hypothalamus, the typical behavioral profile suggests deficits in other parts of the brain also contribute to this complex disorder.

We recently described a mouse strain carrying a gene-targeted *lacZ* insertion into the *Magel2* locus, creating a null *Magel2* allele [Bischof et al., 2007; Kozlov et al., 2007]. *Magel2*-null mice display defective maintenance of their circadian rhythm, reduced hypocretin in the lateral hypothalamus [Kozlov et al., 2007], and reproductive deficits [Mercer and Wevrick, 2009], reflecting a phenotype that is consistent with a hypothalamic defect [Bischof et al., 2007; Kozlov et al., 2007]. They also have substantially reduced total activity measured by 24-hr wheel running but only slightly reduced food intake. This excessive food intake with reduced energy output eventually leads to significantly increased adiposity and a correspondingly altered metabolic profile [Bischof et al., 2007]. To explore a possible role for the *Magel2* gene in learning and behavior, *Magel2*-null mice were subjected to a series of standardized behavioral tests. A high-resolution magnetic resonance imaging (MRI) study of the brain was performed, and brain tissue was analyzed for altered levels of biogenic amines. We now report that loss of *Magel2* affects murine behavior and neurochemistry, and reduces brain volume, particularly in areas involved in reward, emotion, and memory.

MATERIALS AND METHODS

Magel2-Null Mice

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the local Animal Policy and Welfare Committees. *Magel2*-null mice carry a replacement of the *Magel2* gene with a *LacZ* reporter cassette, resulting in complete loss of *Magel2* function in heterozygotes with a paternally derived gene-targeted allele [Kozlov et al., 2007]. The *Magel2*-null mice have been maintained on a C57Bl/6 background for at least 10 generations, and were genotyped by PCR as previously described [Bischof et al., 2007]. Four to eight mice of

each genotype at embryonic day 18 and seven male mice of each genotype at 20–24 weeks of age were used for neurochemical profiling. Unless otherwise noted, behavior tests were performed on adult male *Magel2*-null mice and wild-type control littermates, $n = 5–6$ of each genotype, aged 14–26 weeks, and housed under 12 hr on, 12 hr off lighting at the Centre for Modeling Human Disease in Toronto, Canada. Fat mass, lean mass, and bone mineral content were measured using a PIXImus instrument. These same male mice were MRI imaged at 26 weeks of age. The marble burying, novel object exploration, and grooming tests were performed on male and female age-matched adult mice ($n = 12–20$ of each sex and genotype) in the animal housing unit at the University of Alberta.

Sample Preparation for MRI

Magel2-null mice ($n = 6$) and their wild-type littermates ($n = 6$) were anesthetized at 26 weeks of age with a combination of Ketamine (100 mg/kg, Pfizer, Kirkland, QC, Canada) and Rompun (20 mg/kg, Bayer, Inc., Toronto, ON, Canada) via intraperitoneal injection. A previously described sample preparation protocol for scanning was used with slight modifications [Tyszka et al., 2006]. Thoracic cavities were exposed, and the mice were perfused through the left ventricle with 30 ml of phosphate-buffered saline (PBS, pH 7.4) at room temperature (25°C) at a rate of approximately 1 ml/min. This was followed by infusion with 30 ml of iced 4% paraformaldehyde in PBS. Following perfusion, the heads were removed along with the skin, lower jaw, ears, and the cartilaginous nose tip. The remaining skull structures were post-fixed in 4% paraformaldehyde at 4°C for 12 hr. Following an incubation period of 5 days in PBS and 0.01% sodium azide at 15°C, the skulls were transferred to a PBS and 2 mM ProHance® (gadoteridol, Bracco Diagnostics, Inc., Princeton, NJ) contrast agent solution for at least 7 days at 15°C.

MRI Image Acquisition

A multi-channel 7.0-T MRI scanner (Varian, Inc., Palo Alto, CA) with a 6 cm inner bore diameter insert gradient set was used to acquire anatomical images of brains within skulls. Prior to imaging, the samples were removed from the contrast agent solution, blotted and placed into 13 mm diameter plastic tubes filled with a proton-free susceptibility-matching fluid (Fluorinert FC-77, 3 M Corp., St. Paul, MN). Three custom-built, 14 mm diameter solenoid coils with a length of 18.3 mm and over wound ends were used to image three brains in parallel. Parameters used in the scans were optimized for gray/white matter contrast: a T2-weighted, 3D fast spin-echo sequence, with TR/TE = 325/32 msec, four averages, field-of-view 14 mm × 14 mm × 25 mm and matrix size = 432 × 432 × 780 giving an image with 32 μm isotropic voxels. Total imaging time was 11.3 hr [Henkelman et al., 2006].

Image Processing

The 32 μm isotropic resolution T2-weighted MRI scans were nonlinearly aligned to a three dimensional atlas of the mouse brain with 62 structures identified [Dorr et al., 2008]. This process consisted of an initial step in which all of the MRI scans were

nonlinearly aligned to each other using an unbiased group wise registration algorithm [Kovacevic et al., 2005]. Briefly, rigid body registration was carried out towards a pre-existing image based on the same mouse strain as reported previously [Collins et al., 1994]. All possible pair-wise 12-parameter registrations were then carried out to create an unbiased linear average model of the entire data set. All images were subsequently nonlinearly aligned towards the 12-parameter average. The resulting registered MRIs were resampled and averaged [Collins et al., 1994; Kovacevic et al., 2005]. This iterative procedure was repeated for an additional five generations with increasingly fine deformation grid-point spacings. The end result was that all 12 scans were deformed into exact alignment with each other in an unbiased fashion. This allowed for the analysis of the deformations needed to take each mouse's anatomy into this final atlas space, the goal being to model how the deformation fields relate to genotype. Correspondence with the 3D atlas was obtained by nonlinear alignment of the final stage average MRI with the 40-mouse average MRI upon which the atlas is based [Dorr et al., 2008].

MRI Analysis

Local differences in brain shape related to genotype were assessed by analysis of the deformation fields [Gaser et al., 1999; Nieman et al., 2006]. To reduce random noise and assure normality under the central limit theorem, the transformation data were blurred prior to analysis with a Gaussian kernel with a full width at half maximum of 1 mm, and the logarithm of the Jacobian was computed for univariate statistical comparison at every image point. This statistical analysis results in millions of separate statistical tests. In order to account for an inflated type I error, the False Discovery Rate (FDR) technique was applied [Genovese et al., 2002] with a 10% FDR threshold. The threshold corresponded to an uncorrected *P*-value of 0.00031. The interpretation of these results is that, on average, 10% of the voxels shown as significant will be false positives. The volume for each anatomical structure defined in the atlas was computed for each mouse by integrating the Jacobian of the transformation mapping the atlas image to the image for that mouse. This procedure has previously been shown to provide volume estimates comparable to those obtained by standard stereological methods using tissue sections [Lerch et al., 2008a].

Neurochemical Analysis

Brain regions were dissected from embryonic or adult *Magel2*-null or wild-type littermate control mice, snap frozen on dry ice, then stored at -80°C . Brain samples were processed for HPLC combined with fluorescence detection to measure levels of biogenic amines (noradrenaline (NA), dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), serotonin (5-HT) and amino acids (Trp, Asp, Glu, Asn, Ser, Gln, Gly, Taur, Ala, GABA)) as described [Parent et al., 2001]. Two-way ANOVA with Bonferroni post-test analysis was performed to analyze the effect of genotype on measures of dopamine and serotonin pathways, using GraphPad Prism (San Diego, CA). Immunohistochemistry, confocal microscopy imaging, and cell counts of mouse brain sections was performed

using a primary antibody directed against tyrosine hydroxylase (TH; Chemicon (Millipore) Billerica, MA) or against 5-HT (Immunostar, Hudson, WI) as previously described [Pagliardini et al., 2005].

Behavioral Analysis

Male *Magel2*-null mice and their control littermates were tested in the following order at the Centre for Modeling Human Disease: modified SHIRPA for general health, appearance, and neurological reflexes [Rogers et al., 1997], open field screen, acoustic startle/pre-pulse inhibition screen, tail suspension test, 1-day Rotarod test, learning and memory screen, elevated plus maze, beam test, and 3-day Rotarod test. Before each test, the mice were acclimatized to the testing room for at least 30 min. Test rooms were purpose-designed for behavior testing and controlled for noise, light, humidity, and ventilation. The same observer performed all tests at the Centre for Modeling Human Disease and was blinded with respect to the genotype of the mice. In the open field screen, rearing, time spent in the border area, and time spent in the center area were recorded during the first 5 min. Horizontal and vertical activities were recorded in 5 min intervals for a total of 30 min. The acoustic startle and pre-pulse inhibition screen is generally used to assess sensorimotor gating deficits and was performed as described [Clapcote et al., 2007]. The tail suspension test of depressive traits, the 1- and 3-day Rotarod screen, and the elevated plus maze were performed as described [Soleimani et al., 2008].

The contextual and cued fear conditioning test was performed using the Video Fear Conditioning system (Med Associates, St. Albans, VT) essentially as described [Clapcote et al., 2005]. The apparatus applied foot shocks and auditory tones while recording the activity within the chamber, specifically noting freezing time during the segments of the procedure. Each mouse was acclimatized to the test chamber for 2 min. without any stimulus, to record baseline activity. A 30 sec auditory tone was then applied, accompanied by a foot shock in the last 2 sec of the tone (the conditioning stimulus). Following the shock, the mouse stayed in the chamber for 30 sec before removal to the home cage. The test of contextual memory was performed approximately 24 hr after the conditioning session. The mouse was returned to the test context chamber and freezing behavior measured over 5 min. without any stimulus. The mouse was then placed back into the home cage and allowed to rest for 2 hr before the cued memory test was performed. During this time, the environment within the testing chamber was changed to provide an altered context. The grid floor was covered with a plastic sheet, two pieces of fiberglass were used to create a triangle-shaped chamber, the door was covered with black cardboard and 1% acetic acid was used to wipe down the surfaces. The mouse was placed in the altered context chamber and acclimatized for 3 min. without any stimulus to obtain a day 2 pre-tone baseline measurement of freezing. This was followed by the application of a 3 min. auditory tone, during which time an additional freezing measurement was made. The mouse was then placed back into its home cage.

The custom apparatus for the beam test is made up of two platforms (25 cm \times 35 cm) that are connected with a 90 cm long round beam (18 mm diameter) suspended 50 cm above the floor. One platform was brightly illuminated while the opposite platform

was dark and contained a box providing an enclosed safety area for the mouse. Four consecutive training trials to assist the mouse in navigating the beam were performed on the first day. On the second day, a single trial that recorded the latency to traverse the beam and the number of times the hind feet slipped off was performed.

The one-trial object recognition task [Hammond et al., 2004] was performed in empty rat test cages (26 cm × 48 cm × 20 cm) in which the mouse was habituated for 15 min. Two identical “sample” objects (e.g., a 15 ml plastic tube, a scintillation fluid vial, a syringe barrel) were placed at opposite ends of the cage, ensuring enough room on all sides of the objects for the mouse to walk. The exploration time of the sample objects, defined as the head oriented towards object, 2 cm away or closer, was recorded. The mouse was allowed to explore the sample objects until a total of 38 sec exploration had occurred. The mouse was returned to its home cage for a 5 min recovery period, during which time the test cage was cleaned with 10% ethanol and a familiar sample object and a new “test” object were placed in the test cage. The mouse was returned to the test cage, and the time spent with each object over a 5 min period was recorded. On Day 2, a “sample” object (used in previous day’s test), and a new “test” object were placed in the test cage. The mouse was introduced to the test environment, and time spent with each object over a 5 min period was again recorded. Control mice typically spend more time exploring the test object over a retention time of minutes to days, suggesting that they recognize the familiar object. For the marble burying analysis, mice were tested individually in clear mouse cages with 5 cm of bedding, in the dark. Each mouse was allowed a minimum of 2 hr to acclimate to the testing room and cage, after which the mice were removed while testers placed twelve clear glass marbles (0.6 cm in diameter—three rows of four) in each cage. Mice were returned to the testing cage and were removed to their home cages after 3 min. The number of marbles that were not buried was recorded. Marbles were considered buried if covered at least 75% with bedding [Deacon, 2006]. The grooming test was performed in a clean mouse cage with standard bedding. The grooming pattern (direction, length of time) was observed when the mouse was placed in the new cage, and after a gentle misting with water [Kalueff et al., 2007].

Behavioral data were analyzed statistically by *t*-test except the horizontal and vertical activity profile, the 3-day Rotarod test, and the fear-conditioning test, which were analyzed by two-way ANOVA with Bonferroni post-tests, using GraphPad Prism. Probabilities <0.05 were deemed significant.

RESULTS

Expression of *Magel2* in the Adult Mouse Brain

We previously outlined expression of *Magel2* in the embryonic brain by RNA in situ hybridization [Lee et al., 2000, 2003], and described expression in the hypothalamus, lateral septal complex, and the bed nucleus of the stria terminalis by staining for the lacZ reporter gene in brain from *Magel2*-null adult mice [Kozlov et al., 2007]. To refine the regions of *Magel2* expression, we examined the Allen Brain Atlas, an on-line resource that catalogues RNA in situ hybridization profiles of the adult mouse brain [Lein et al., 2007]. Highest levels of expression were observed in the hypothalamus,

with expression also seen in the medial septal nucleus, lateral septal complex and bed nucleus of the stria terminalis. Single cells in the outer region of the external granule layer of the cerebellum also express *Magel2*, and expression was present broadly throughout the pons and the medulla, and in the nucleus of the solitary tract (Suppl. Fig. 1).

General Behavior and Health of *Magel2*-Null Mice

No overt differences in the acquisition of developmental milestones (e.g., physical development, rooting reflex, righting reflex, forelimb/hind limb grasping, or locomotor behavior [Crawley, 2007]) were noted in *Magel2*-null mice compared to control littermates from birth to 3 weeks of age, suggesting adequate muscle tone. There was a 5–10% reduction in pre-weaning body weight similar to that previously reported [Bischof et al., 2007]. All adult mice were also healthy, and *Magel2*-null mice performed normally in a formal screen for general health and neurological reflexes (SHIRPA screen [Rogers et al., 1997]). Adult body composition abnormalities were consistent with our previous report, with increased fat mass (131% of control, $P < 0.01$) and decreased lean mass (92% of control, $P < 0.005$), measured by a PIXI-mus small animal densitometer at 26 weeks. Decreased bone mineral content (87% of control, $P < 0.05$) was present, but total body mass was not significantly different between genotypes. Gait and postural abnormalities were occasionally observed in the home cages, with *Magel2*-null mice displaying elevated head position during ambulation and hopping movements with both hind limbs together, movements that were never observed in wild-type mice.

Neuroanatomical Abnormalities in *Magel2*-Null Mice Measured by MRI

No gross abnormalities in sections of the *Magel2*-null mouse brains were detected by Nissl staining (not shown). To examine the brain more finely and without the confounding effect of removal from the skull, very high resolution MRI combined with computer analysis was performed. MRI analysis revealed reduced brain volume in the mutant mice (3.4% smaller than control brain, $P < 0.01$). We used a statistical map of the Jacobian determinant that illustrates the expansion and contraction of tissue based on genotype, to find regions of significant change. In order to account for an inflated amount of false positive findings due to the number of statistical tests employed, the FDR technique was applied [Genovese et al., 2002] with a 10% FDR threshold. This means that on average, 10% of the structures that show significant volumetric change, will be false positive (zero or one structure in our case). The following regions have a 10% or less chance of being a false positive: the parieto-temporal lobe of the cerebral cortex (representative single coronal slices in Fig. 1), the amygdala, the dentate gyrus of the hippocampus, and the nucleus accumbens, which are regions of moderate to high *Magel2* expression as described above. The mean difference in volume for these regions was found to be significantly smaller by 4–5% in *Magel2*-null mice. Notably, no volumetric differences were present in the hypothalamus, which typically has maximal *Magel2* expression, suggesting that loss of *Magel2* expression in the hypothalamus does not cause structural changes. The

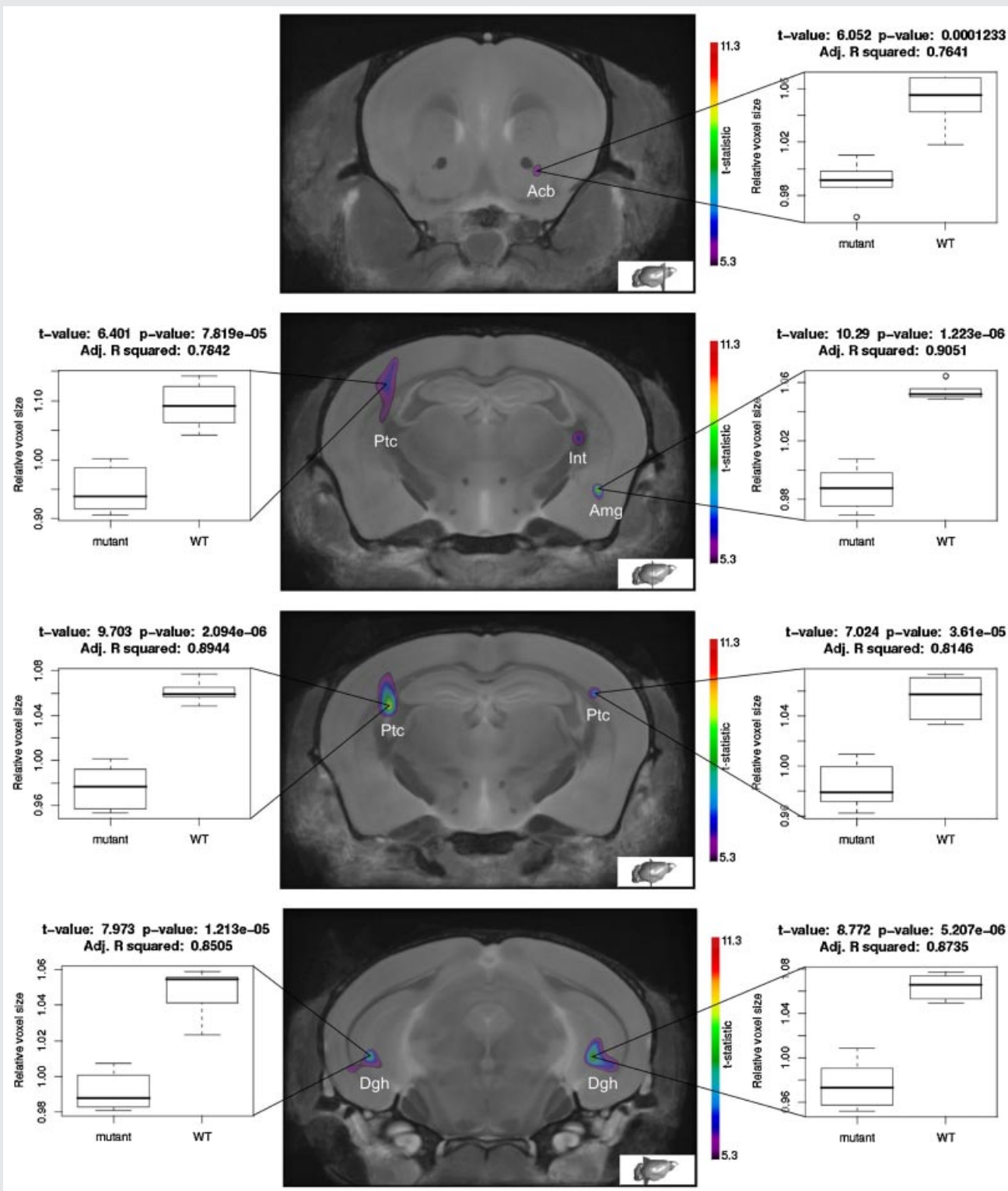


FIG. 1. High resolution magnetic resonance imaging of the *Magel2*-null adult brain. The per-voxel results of tests of the Jacobian maps (tissue compression/expansion) are shown as composite images of four coronal sections of the *Magel2*-null (mutant) brain compared to wild-type control (WT) brain. All colored voxels are significant with a false discovery rate of 10% ($n = 6$ of each genotype). The coronal level of each section is pictorially illustrated in the bottom right corner. Acb, nucleus accumbens, Amg, amygdala, Dgh, dentate gyrus of the hippocampus, Int, internal capsule, Ptc, parieto-temporal lobe of the cortex. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

corpus callosum and the olfactory bulbs were also reduced in size by 5%. No other regions of the brain showed statistically significant changes in size. The pituitary gland was normal in size and location, as was the pineal gland, which forms part of the circadian rhythm output pathway (data not shown).

Neurochemical Analysis of Brain regions in *Magel2*-Null Embryos and Adult Mice Reveals Altered Serotonin and Dopamine Levels

To examine the neurochemical profile (biogenic amines and their metabolites, and amino acids) of various brain regions, high performance liquid chromatography (HPLC) with fluorimetric detection was performed (Table I). Brain lysates were prepared from the cortex and cerebellum of brains from embryonic day 18 embryos and from the following brain regions of adult male *Magel2*-null and age-matched control mice: olfactory bulb, prefrontal cortex, cortex, hypothalamus, and cerebellum. No significant neurochemical differences between *Magel2*-null and control E18.5 brain samples were detected by two-way ANOVA (Table I). A more striking neurochemical imbalance was noted in brain samples from adult male *Magel2*-null mice when analysis was performed for compounds in the catecholamine pathway (effect of genotype ($F(1,144) = 9, P < 0.003$)) and the indolamine pathway (effect of genotype ($F(1,108) = 73, P < 0.0001$)). On post hoc analysis, the serotonin concentration was decreased compared to control in the prefrontal cortex, cortex, and the hypothalamus, while the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) was

lower in the cortex and the hypothalamus in male mice. Brain samples from adult female *Magel2*-null mice also demonstrated statistically significant reductions in serotonin concentrations to 71% (cortex) and 60% (hypothalamus) of control levels respectively. The concentration of dopamine was decreased in adult male *Magel2*-null hypothalamus, but was not significantly different from control in other regions of the brain or in the female mice. There were no neurochemical abnormalities in *Magel2*-null cerebellum or olfactory bulb samples. Turnover rates for the catecholamine and indolamine pathways (NA/DA, DOPAC/DA, HVA/DA, 5-HT/Trypt, 5-HIAA/5-HT) were similar in the two genotypes. The concentration of various amino acids was also similar between the control and *Magel2*-null brain samples (data not shown).

The reduced concentration of serotonin or dopamine could be caused by a reduction in the number of dopaminergic or serotonergic neurons in the *Magel2*-null mouse brains. To examine this possibility, we first examined the location and number of dopaminergic neurons (immunoreactive with an antibody against TH and visualized by confocal microscopy) in the hypothalamus in adult and *Magel2*-null male mouse brain sections. We found a comparable number of positively staining neurons in both genotypes, and no differences in the appearance of the TH-positive neurons groups in the pre-optic region, the medial basal region, or the medial dorsal region of the hypothalamus (A11–A15 dopaminergic cell groups, Suppl. Fig. 2). Serotonergic cell groups are primarily located in the brain stem, a region of low *Magel2* expression. Examination of the location and number of neurons immunohistochemically labeled

TABLE I. Biogenic Amine Concentrations in Brain Regions From *Magel2*-Null and Wild-Type Control Littermate Mice

	Catecholamine pathway				Indolamine pathway		
	DA	NA	DOPAC	HVA	TRYPT	5HT	5HIAA
E18 cortex							
WT	113 ± 7	83 ± 2	16 ± 1	24 ± 1	15.8 ± 1.5	120 ± 14	256 ± 8
<i>Magel2</i>	97 ± 12	93 ± 7	22 ± 2	30 ± 2	10.8 ± 0.6	109 ± 12	286 ± 21
E18 cerebellum							
WT	52 ± 6	123 ± 10	76 ± 23	45 ± 5	16.6 ± 1.9	195 ± 19	678 ± 77
<i>Magel2</i>	54 ± 9	116 ± 15	21 ± 3	28 ± 1.2	9.2 ± 1.4	168 ± 32	335 ± 78
Adult prefrontal cortex							
WT	2658 ± 146	376 ± 24	723 ± 58	581 ± 33	3.2 ± 0.2	441 ± 21	250 ± 30
<i>Magel2</i>	2483 ± 166	359 ± 20	565 ± 93	464 ± 32	3.2 ± 0.2	369 ± 10	218 ± 20
% of WT, <i>P</i> -value						84%, <i>P</i> < 0.01	
Adult cortex							
WT	873 ± 58	375 ± 11	176 ± 11	211 ± 11	4.3 ± 0.1	377 ± 13	295 ± 16
<i>Magel2</i>	912 ± 52	317 ± 14	189 ± 21	206 ± 18	4.1 ± 0.3	282 ± 10	225 ± 9
% of WT, <i>P</i> -value						75%, <i>P</i> < 0.001	76%, <i>P</i> < 0.01
Adult hypothalamus							
WT	783 ± 137	587 ± 27	196 ± 42	328 ± 41	4.8 ± 0.5	528 ± 22	520 ± 19
<i>Magel2</i>	472 ± 75	557 ± 25	132 ± 24	246 ± 22	4.4 ± 0.6	372 ± 18	421 ± 13
% of WT, <i>P</i> -value	60%, <i>P</i> < 0.05					70%, <i>P</i> < 0.001	81%, <i>P</i> < 0.001

Samples are from male wild-type control (WT) and *Magel2*-null [*Magel2*] mice at embryonic Day 18 (E18, $n = 8-12$ per genotype) or at 20–24 weeks of age (adult, $n = 7$ per genotype). Concentrations (mean ± SEM) are expressed as ng/g of tissue except for tryptophan, which is expressed as µg/g tissue. Two-way ANOVA detected a difference between genotypes in the adult samples in both the catecholamine [$F(1,144) = 9, P < 0.003$] and indolamine [$F(1,108) = 73, P < 0.0001$] pathways. Bonferroni post-test *P* values are provided only where statistically significantly different between genotypes at $P < 0.05$.

DA, dopamine; NA, noradrenaline; DOPAC, dihydroxyphenylacetic acid; TRYPT, tryptophan; HVA, homovanillic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.

with an anti-5-HT antibody revealed no differences in the location or number of serotonergic neurons in the cell groups in the brain stem (Suppl. Fig. 2). The quantitative reduction in dopamine and serotonin content in the hypothalamus of *Magel2*-null mice therefore does not appear to be consequent to neuronal loss, consistent with our finding that there is no volumetric reduction in either the hypothalamus or the brain stem measured by structural MRI.

Magel2-Null Mice Become Less Active Over Time in the Open Field, But Have Normal Balance and Strength

We determined whether behavioral abnormalities existed in adult *Magel2*-null mice that could be quantified on formal testing. Motor activity was assessed in an open field test, measuring rearing, exploratory, and fear-related behavior (percent time in the center and the average time per crossing) in the first 5 min of the test. Off-wall rearing was increased in the *Magel2*-null mice (Fig. 2A, $P < 0.03$), but on-wall rearing, time in the center and time per crossing were not different between genotypes (Fig. 2A and data not shown). As expected from our previous study that measured home cage wheel running activity [Kozlov et al., 2007], horizontal activity in the open field test was reduced (Fig. 2B, $P < 0.001$), and we also observed reduced vertical activity (Fig. 2C, $P < 0.002$). Moreover, while the control mice slightly decreased their levels of horizontal activity and increased their vertical activity over the 30 min interval, the *Magel2*-null mice became less active in both dimensions during the test. Overall, there was a significant effect of genotype on the results of the open field test ($F(1,48) = 16$, $P < 0.0002$), with the *Magel2*-null mice only about half as active as control in either the horizontal or vertical dimensions by the end of the 30 min test period.

We used the accelerating Rotarod screen to test motor coordination and balance. The *Magel2*-null mice had a 50% increase in the latency to fall off the Rotarod in a single test (Fig. 2D, $P < 0.02$), which was unexpected given their reduced muscle mass and overall reduced activity. The same mice were then tested over a 3-day period to assess motor function and learning. As in the 1 day test, the *Magel2*-null mice had increased latency to fall from the Rotarod over the 3 days (Fig. 2E, $F(1,30) = 15$, $P < 0.0006$). For both genotypes, there was increased latency on day 3 compared to Day 1 ($P < 0.05$), and this motor learning was not different in the *Magel2*-null mice compared to control. As a further test of motor function and motivation, the time for each mouse to travel along a narrow beam to reach an enclosed safety platform was measured. There was no difference between genotypes in the traversing time, nor in the number of times the hind feet slipped off the beam (data not shown). A tail suspension test is designed to test for depression-like behavior, and is based on the observation that mice subjected to an inescapable situation will spend more time immobile when displaying depressive behavior, but also secondarily measures strength as the mouse struggles against being suspended. The latency to first immobile period after suspension was not different between genotypes, nor was the amount of time immobilized at 6 min (data not shown). Taken together, the tail suspension test, the

Rotarod tests, and the beam test, and the increase in off-wall rearing in the first 5 min of the open field suggest that motor ability per se is not compromised in the *Magel2*-null mice. These tests suggest that the measurably reduced activity after habituation is not caused by deficient motor function, but may instead represent passive or anxious behavior in the open field.

Tests of Anxiety and Learning

The elevated plus maze is a more specific test of anxiety in a novel environment. The time spent in the open or closed arms, time spent on the end of the open arms and on the central platform, time spent freezing in the open and freezing in the center, the number of passes between closed arms, the number of risk assessments, and the number of head dips were all measured, but no significant differences between genotypes were detected (data not shown).

We then used a Pavlovian fear-conditioning test that measures percent of time spent with total lack of movement (freezing) after an aversive stimulus, and the learning associated with this stimulus. This test also models anticipatory anxiety, and requires a combination of amygdalar and hippocampal function. Each mouse was acclimatized in a test context, then a 30 sec auditory tone was used as the conditioned stimulus paired with an aversive unconditioned stimulus, in this case a 2 sec mild foot shock at the end of the tone. After conditioning, either the test context or the tone typically elicit a state of fear even in the absence of the foot shock, in a normal mouse. This fear is manifested as freezing, and is used to measure learning when assessed after 24 hr.

The *Magel2*-null mice tended towards an increased amount of time spent freezing during the baseline measurement on Day 1 ($1.2 \pm 0.7\%$ for control mice, $12.4 \pm 5.7\%$ for the *Magel2*-null mice, $P < 0.09$, all values expressed as mean \pm standard error of the mean (SEM), Fig. 3A). The wild-type mice exhibited an increased freezing rate after the tone (4.9-fold compared to baseline, $P < 0.05$) and after the foot shock (13.2-fold compared to baseline, $P < 0.06$), as expected. In contrast, there was no significant change from the already high baseline level of freezing in the *Magel2*-null mice, either after the tone or after the foot shock (Fig. 3A). The amount of freezing after the tone also remained higher in the *Magel2*-null mice compared to wild-type control (percent freezing $5.7 \pm 2.6\%$ in control mice, $23.8 \pm 7.5\%$ in *Magel2*-null mice, $P < 0.05$). After 24 hr (Day 2), each mouse was placed back into the test context used on day 1 and freezing was measured (context in Fig. 3A). Both genotypes increased freezing when placed in the test context on day 2 compared to their day 1 baseline measurement, indicating normal contextual discrimination, although the mutant mice had significantly higher absolute freezing rates ($P < 0.01$) (Fig. 3A). Two hours later, freezing was measured in an altered context, both before (pre-tone) and after the application of a tone, to measure learning related to the cued conditioning stimulus. Similarly to the result from the baseline measurement on Day 1, the *Magel2*-null mice trended towards increased freezing in the altered context on Day 2 (pre-tone, $1.6 \pm 0.6\%$ for control mice, $6.8 \pm 2.3\%$ for the *Magel2*-null mice, $P < 0.07$). Freezing to the tone was increased compared to pre-tone freezing in both genotypes, indicating normal cued conditioning, albeit with higher absolute rates of freezing in the *Magel2*-null mice ($P < 0.001$). A two-way ANOVA analysis of all six

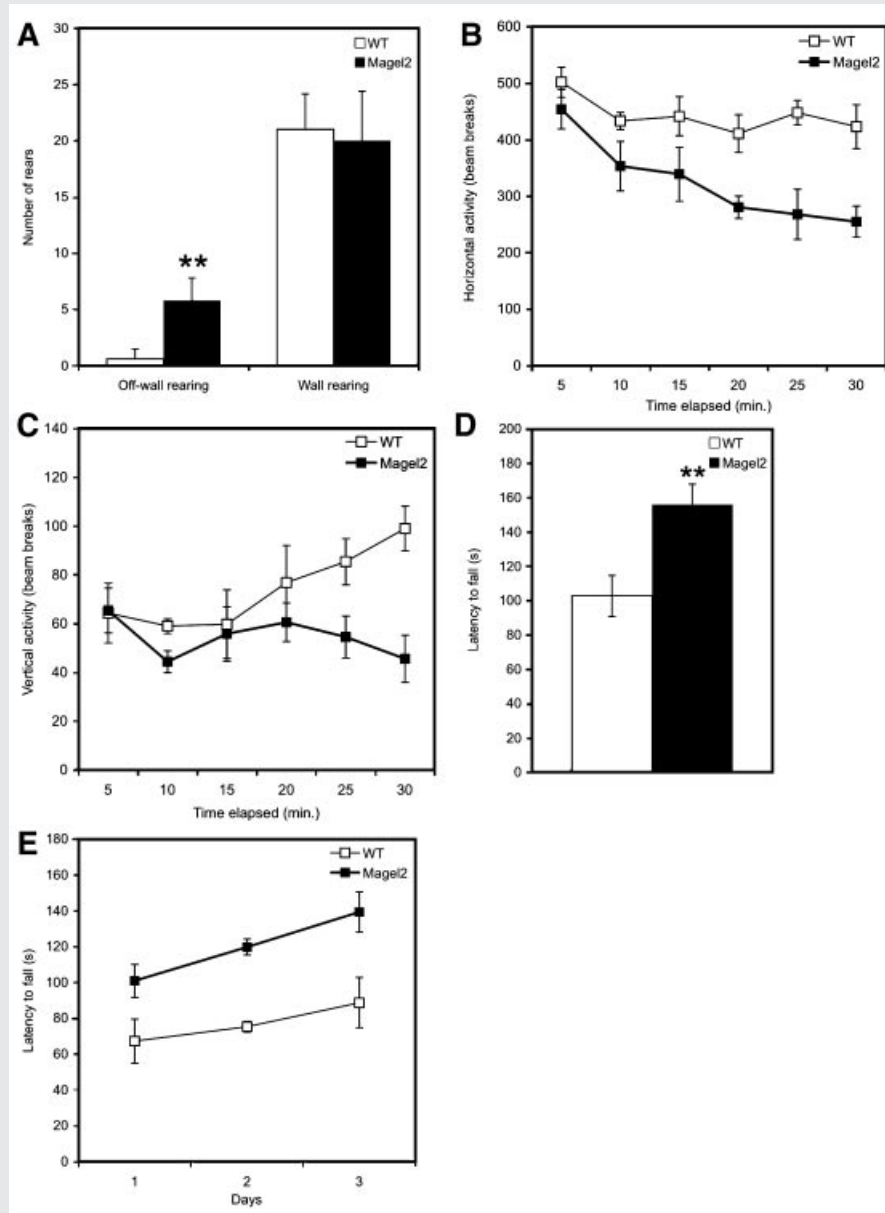


FIG. 2. Motor function testing. A: Measurements of rearing either off the walls or on the walls in the first 5 min of the open field test. Data are expressed as mean \pm SEM. Increased off-wall rearing in the *Magel2*-null [*Magel2*] mice versus wild-type control (WT) was noted; ** $P < 0.05$. B,C: Horizontal and vertical activity in an open field environment over a period of 30 min, measured as number of beam breaks over 5 min intervals, are both reduced in the *Magel2*-null mice [two-way ANOVA with main effect of genotype, $P < 0.0002$]. D: Latency to fall from an accelerating Rotarod during a single test is significantly increased in the *Magel2*-null mice; ** $P < 0.05$. E: Latency to fall from an accelerating Rotarod increases for both genotypes over three consecutive days indicating normal motor learning. Latency to fall is significantly increased in the *Magel2*-null mice [two-way ANOVA, main effect of genotype $P < 0.0006$].

data sets with genotype as the between factors variable revealed a significant effect of genotype on measures of freezing ($F(1,48) = 36$, $P < 0.0001$). Overall, this test indicates increased anxiety in response to novel environments or noise, but no measurable abnormality in the learning, memory, or emotional components required for the *Magel2*-null mice to remember the association between either the context or tone and the foot shock on Day 2.

Magel2-Null Mice Display Altered Behavior in Novel Environments

As a further test to discriminate anxiety from learning, we performed a set of tests that measure reactions to novel objects in an independent cohort of male and female mice that were naïve to behavioral testing. In a one-trial object recognition task, each

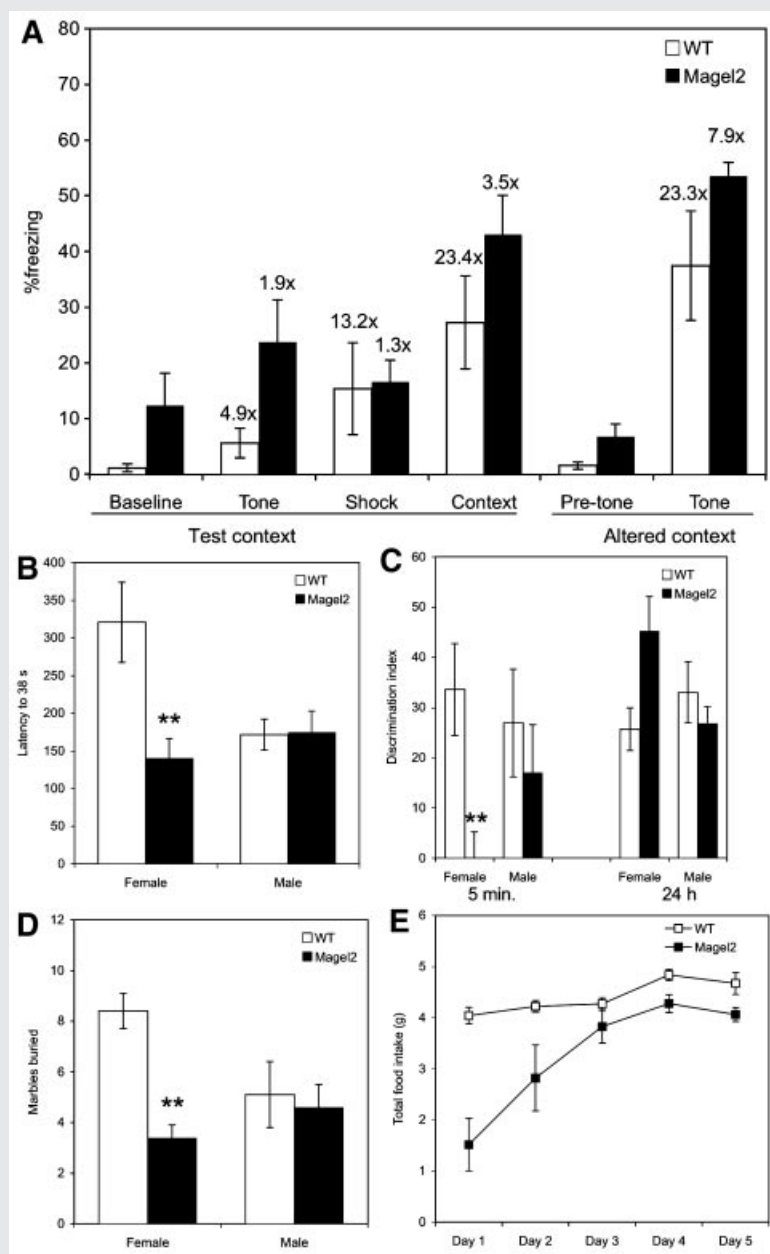


FIG. 3. Fear conditioning test and novel object scenarios reveal abnormal behavior in *Magel2*-null mice. **A:** *Magel2*-null mice freeze more than wild-type (WT) control mice in the test context at baseline and after the auditory tone, but have similar freezing rates after the foot shock. On Day 2, freezing in the familiar test context was not different between genotypes, but in contrast, *Magel2*-null mice freeze more than control at baseline in the altered context, pre-tone. Also on Day 2, the net amount of freezing in the novel context either before [pre-tone] or after [tone] the auditory tone was not different between genotypes. Data are expressed as mean \pm SEM. Two-way ANOVA detected a main effect of genotype across the six freezing measurements ($P < 0.0001$). The fold change [x] in freezing during fear conditioning is also presented as a ratio of the means. Fold changes in the test context [tone, shock, day 2 context] are compared to baseline. Fold changes in the altered context after the tone are compared to the pre-tone measurement. *Magel2*-null mice show a blunted fear reaction to either the test context or to the tone. **B:** Female *Magel2*-null display increased exploratory behavior when placed in a cage with two novel objects, with decreased latency to reach 38 sec total exploration time. Data are expressed as mean \pm SEM, $**P < 0.05$. **C:** Control mice of both sexes and male *Magel2*-null mice spend more time with the novel object than with the familiar object, measured after 5 min and after 24 hr, and presented as a discrimination index (the difference between the time with the novel object and the time with familiar object divided by the total time). *Magel2*-null female mice show no preference for a novel object after 3 min (DI = 0, $**P < 0.05$). **D:** Male mice of both genotypes buried similar numbers of marbles over the three min. test interval. In contrast, female *Magel2*-null mice buried significantly fewer marbles than controls; $**P < 0.05$. **E:** Control mice consume slightly less food during their first two days in an Accuscan chamber with powdered food, but increase food consumption to normal levels by day 3 in the chamber. In contrast, *Magel2*-null mice have substantially reduced food intake when initially placed in the Accuscan chamber, and never attain normal levels of food intake.

mouse was removed from the home cage and acclimatized to the test cage in which two objects (e.g., plastic 15 ml tube, 10 ml syringe) were placed. The total length of time needed to accumulate 38 sec of active object exploration was recorded. *Magel2*-null female mice explored the objects more actively than their control littermates, accumulating 38 sec of exploration time within 141 ± 26 sec, while the control female mice required twice as long to accumulate the total exploration time (321 ± 53 sec, $P < 0.02$) (Fig. 3B). In contrast, the male mice showed no genotype-specific difference in exploration time. After removal of the mouse to the home cage for 5 min, one of the objects was replaced with a novel object of similar size, and the mouse was then returned to the test cage for 5 min. Wild-type mice typically spend 70% of their time with the novel object and 30% with the familiar object under this paradigm [Hammond et al., 2004]. These data are presented as a discrimination index (DI), the difference between the time with the novel object and the time with familiar object divided by the total exploration time. In this 5 min interval, the total time spent exploring was no longer different between genotypes for either sex, but there were differences in the rate of exploration of the novel versus the familiar object. While male and female wild-type mice and male *Magel2*-null mice preferred the novel object with this predicted preference ratio (e.g., female control mice $67 \pm 5\%$, significantly different from 50%, $P < 0.007$, $DI = 34 \pm 9$), female *Magel2*-null mice showed no preference for the novel object (preference $53 \pm 6\%$, not different from 50%, $DI = 0 \pm 5$) (Fig. 3C). The test was repeated after 24 hr, with one of the objects identical to one from the previous day and the second a completely novel object. As in the initial test period, *Magel2*-null female mice spent more total time exploring the objects ($P < 0.03$). All classes of mice preferred the novel object (significantly different from 50%, $P < 0.01$ for all sex/genotypes, Fig. 3C). One interpretation of this result is that object memory is intact in *Magel2*-null mice, but that *Magel2*-null female mice are averse to the new object in the 5-min test. As this task requires memory in addition to motor function and motivation [Crawley, 2008], an alternative hypothesis is that short-term memory is impaired but long-term memory is preserved in the female mutant mice.

We used a marble-burying test to further assess behavior with novel objects, without a confounding requirement for memory. Mice will normally exhibit digging behavior when placed in a cage with marbles on the surface of the bedding, and a number of psychotropic compounds inhibit or abolish this behavior [Deacon, 2006]. It remains controversial whether marble burying simply represents a natural behavior in mice, or whether increased marble burying represents an anxious state [Deacon, 2006]. The number of marbles buried in a 3 min period was measured for male and female mice of both genotypes. There was no difference between genotypes in the male mice, but female *Magel2*-null mice buried significantly fewer marbles than the control mice ($P < 0.0001$, Fig. 3D).

Our hypothesis that *Magel2*-null mice tend to avoid novel objects and are anxious in novel environments is supported by observations we made during a previous feeding study. In this experiment, male mice were placed in a chamber with a powdered standard chow dispenser that measured hourly food consumption over 5 days [Bischof et al., 2007]. To avoid confounding the results while the mice acclimatized to the feeding system, only Days 3, 4,

and 5 after placement in the new cage were used for calculation of food consumption, which we reported was reduced to 88% of the control amount [Bischof et al., 2007]. In a re-analysis of the food intake data from days 1 and 2, the *Magel2*-null mice were observed to consume only 43% of the control amount on the day 1 (*Magel2*-null 1.7 ± 0.5 g compared with control, 4.0 ± 0.2 g, $P < 0.0005$) with three of these eight *Magel2* mice consuming less than 0.08 g of food over 24 hr (Fig. 3E), and mutant mice consumed only 66% of wild-type levels on Day 2. These results suggest avoidance of the novel food source or anxiety in the new type of cage.

Additional Tests of Behavior

Mice normally exhibit self-grooming behavior, and pathological self-grooming or excessive grooming of cage mates has been interpreted as evidence of obsessive-compulsive tendencies in mice [Kalueff et al., 2007]. We examined grooming behavior either under unstimulated conditions in the home cage, or by individually spraying the mice with a fine mist of water from a hand spray bottle. Normal head to tail grooming behavior was observed in mice of both genotypes. Finally, there were no differences between genotypes in the pre-pulse inhibition test that provides a physiological measurement of sensorimotor gating (data not shown).

DISCUSSION

Magel2 belongs to the MAGE family of proteins, and is most closely related to *MAGED1/NRAGE* and *necdin*. Studies in cell culture and in mice have implicated *necdin* in neural differentiation and cell cycle exit, and structural and function deficits in the nervous system have been identified in *necdin*-null mice [Muscatelli et al., 2000; Takazaki et al., 2002; Ren et al., 2003; Lee et al., 2005; Tennesse et al., 2008]. *MAGED1/NRAGE* inhibits cell cycle exit and facilitates p75 neurotrophin receptor-mediated apoptosis in neuronal cells [Salehi et al., 2000]. While *necdin* and *MAGE-GI* were shown to have activities in p75 neurotrophin receptor and E2F1-dependent pathways, similar activities could not be detected for *MAGEL2*, perhaps because only 490 amino acids of the 525 amino acid predicted protein [Lee et al., 2000] were included in the epitope-tagged expression construct [Kuwako et al., 2004]. Although a cellular role for *Magel2* has not been determined, we previously described abnormalities in maintenance of behavioral circadian rhythm, reduced total activity, and reduced food intake in *Magel2*-null mice [Bischof et al., 2007; Kozlov et al., 2007]. We also described decreased weight gain before weaning and increased gain after weaning leading to increased adiposity and reduced lean muscle mass in adult mice. We have now characterized the neuroanatomy, neurochemistry, and behavior in mice null for *Magel2* and found deficits in all three areas.

Magel2-Null Mice Have Reduced Brain Volume and Reduced Neurotransmitter Levels in Discrete Regions of the Brain

Subtle changes in regional brain volume have been described in a variety of congenital and progressive genetic disorders, most

prominently in human and mouse studies of schizophrenia [Clapcote et al., 2007; van Haren et al., 2008]. In a recent study of mice with heritable mutations that display behavioral symptoms, 87% of these strains showed neuroimaging abnormalities including volumetric changes using high resolution MRI [Nieman et al., 2007]. In keeping with this finding, *Magel2*-null mice have brain abnormalities that we detected by MRI. In particular, we noted a 4% bilateral reduction in cortical volume in distinct regions of the *Magel2*-null adult mouse brain, including focused regions in the parietal-temporal lobes, and in the dentate gyrus of the hippocampus underlying the medial temporal lobe of the cortex. Notably, there were no anatomical abnormalities in the hypothalamus in *Magel2*-null mice, suggesting that the circadian rhythm deficiency in these mice is not structural in origin. These regions of volumetric reduction in the mouse are also regions of moderate to high levels of *Magel2* expression. A significant volumetric change with a magnitude of 4–5% is not atypical in mutant mice, and for example changes of a similar magnitude have been observed using high resolution MRI in a mouse model of Huntington disease [Lerch et al., 2008b] and between sexes in the C57BL/6 mouse strain [Spring et al., 2007]. Ventriculomegaly was previously observed in *Ndn*-null mouse embryos [Lee et al., 2005], but in contrast, brain ventricles were normally sized in *Magel2*-null mice. Notably, volumetric changes in specific brain regions that are of comparable magnitude to that seen in the *Magel2*-null mouse brain (~5%) have been described in studies of children with autism (frontal lobe enlargement of 5–10% [Amaral et al., 2008]), attention deficit hyperactivity disorder (cerebrum reductions of 3–5% [Krain and Castellanos, 2006]) and early onset schizophrenia (e.g., progressive 5–10% reduction in the prefrontal cortex [Toga et al., 2006]) suggesting that this extent of volumetric change has neurologically based consequences to mammalian behavior.

We identified neurochemical abnormalities in brain samples from adult *Magel2*-null mice, most notably reduced concentration of serotonin and its metabolite 5-HIAA to 70–80% of control levels in the prefrontal cortex, cortex and hypothalamus. We found no difference in the abundance or location of the serotonergic neuron clusters that are primarily located in the raphe nuclei of the brainstem. The rostral serotonergic group projects axons throughout the fore- and mid-brain, where reduced serotonin content was measured in the mutant mice in this study. In addition, dopamine was reduced to 60% of control values in the adult *Magel2*-null hypothalamus, although the number and location of dopaminergic neurons was not different between genotypes. Dopamine producing neurons are present in the arcuate nucleus of the hypothalamus where they project axons to the median eminence to stimulate hormone secretion from the anterior pituitary. In particular, they control the secretion of the reproductive hormone prolactin from the pituitary, and dopaminergic pathways in the hypothalamus are also critical to the regulation of growth hormone releasing hormone action on the release of growth hormone from the anterior pituitary [Garcia-Tornadu et al., 2006]. Dopamine concentrations were not altered in other brain regions suggesting a specific effect of the *Magel2* mutation on hypothalamic dopaminergic neurons.

It is difficult to establish a cause and effect relationship between neurochemical imbalances and behavior in mice, particularly as

total levels combine the intracellular and extracellular pools of the neurotransmitters. For example, many studies implicating serotonin in mood and behavior use surrogate markers, such as 5-HT or 5-HIAA levels in cerebrospinal fluid or platelets, examine the activation of serotonin receptors after pharmacological intervention, or the effects of depletion of the serotonin precursor tryptophan on behavior. Nonetheless, many studies have linked altered serotonergic and dopaminergic pathways with psychiatric disorders, most notably depression, anxiety, and self-injurious or obsessive behavior [Hyman, 2007]. Serotonin pathways have also been implicated in eating disorders [Kaye et al., 2005], and serotonin is intimately involved in food intake and energy balance at least in part through modulation of the activity of hypothalamic neurons, particularly through melanocortin-dependent pathways [Heisler et al., 2006]. Serotonin, brain-derived neurotrophic factor, and the insulin-like growth factor cooperate in the regulation of energy metabolism and stress response in cells and whole organisms, through their roles as neurotrophic factors in the central nervous system [Mattson et al., 2004]. Interestingly, mice deficient for brain-derived neurotrophic factor exhibit abnormal behaviors, including obesity, anxiety, and aggression, and also significantly reduced levels of 5-HT and 5-HIAA in the adult cortex [Gaspar et al., 2003]. Further studies are needed to determine whether there is a concurrent disruption of the insulin-like growth factor or brain-derived neurotrophic factor pathways in *Magel2*-null mice that could alter the balance of growth and survival of neurons.

***Magel2*-Null Mice Are Hypoactive and React Abnormally to Novel Environments**

We performed assays in *Magel2*-null mice designed to measure anxiety-like behavior, locomotion, balance, neuromuscular function, learning, and memory. Behavioral tests in animal models can provide surrogate markers for normal or pathological human behavior. In transgenic animal studies, interacting deficits in different processes can influence performance in behavior tests [Crabbe and Morris, 2004; Tecott and Nestler, 2004]. For example, a Rotarod test meant to assay motor function and motor learning also assesses vision, motivation not to fall, grip strength, and balance. The effects of reduced total activity, reduced muscle mass, and blunted circadian rhythm on the behavior tests that we performed also need to be considered in our analysis.

Balance and strength were not impaired by the *Magel2* mutation, as evidenced by normal or improved function in rearing in the open field, time to cross in the beam test, increased latency to fall from the Rotarod, and equivalent time spent struggling in the tail suspension test. One interpretation of the increased latency to fall from the Rotarod is that the *Magel2*-null mice have normal strength and balance, as supported by the other tests of motor function, but have increased motivation not to fall, consistent with their abnormal reaction to other novel environments. We did however observe a significant reduction in open field activity in *Magel2*-null mice, consistent with previous findings that used running wheels to monitor 24 hr activity of *Magel2*-null mice [Kozlov et al., 2007]. This reduction to about 50% of control activity, accompanied by only a 10% reduction in food consumption, causes the obesity in adult *Magel2*-null mice.

We found no evidence for learning or long-term memory deficiencies in the 3-day Rotarod test, the 24 hr novel object preference test, or the 24 hr fear-conditioning test. There was an increase in freezing in the *Magel2*-null mice on day 2 of the fear conditioning test, suggesting they are not grossly impaired in amygdalar or hippocampal functions required for conditioned learning over 24 hr. The interpretation of learning in the fear-conditioning test was complicated by the high baseline freezing rates in the *Magel2*-null mice, which led to a smaller fold increase in freezing either to the context or to the tone on Day 2.

The conditioned fear test revealed a significant difference between genotypes not related to learning or memory: male *Magel2*-null mice have increased time spent freezing under baseline conditions, indicating increased anxiety in the test chamber. It is unlikely that reduced activity accounts for this increase in freezing during the 2 min baseline measurement, as there was only minimal difference in horizontal or vertical activity between genotypes in the first 5 min of the open field test. Rather, there was a progressive decline in activity of the *Magel2*-null mice over the following 25 min in the open field. In a test used as a proxy for anxiety in rodents, we found no inter-genotype difference in marble burying activity in male mice, nor was there any difference in the time male mice spent with objects in the cage. In contrast, female *Magel2*-null mice spent more time exploring objects placed in a cage, did not display novel object preference when placed back into the test chamber after a 5 min interval, and were less likely to bury marbles placed on the surface of the bedding. In summary, the object-based tests with the female mice suggest a combination of poor short-term memory for novel objects and decreased motivation to manipulate objects in the cage. Interestingly, sex-specific differences in behavioral responses to novel objects were also observed in a serotonin-depletion mouse model of developmental brain disorders [Hohmann et al., 2007]. Further studies of anxiety-like behavior in these mice using drugs that interact in dopaminergic and serotonergic pathways will be necessary to fully understand the extent of the behavioral deficiencies that results from loss of *Magel2*.

Abnormalities of Brain and Behavior: Comparison With PWS

In PWS, psychiatric symptoms often develop during childhood and can include mood instability, obsessive-compulsive disorder, autism spectrum disorder, cognitive rigidity, anxiety, and addictive behavior towards to food and other substances [Cassidy and Morris, 2002; Vogels et al., 2004; Soni et al., 2007, 2008]. Structural changes in the brain have been detected in individuals with PWS by MRI, and include enlarged ventricles (100% of individuals tested), decreased brain volume in specific regions, particularly the parietal and occipital lobes (all children over age 5 and adults), and polymicrogyria (60%) [Miller et al., 2007a], while a thin corpus callosum and reduced myelination are occasionally noted [Miller et al., 1996, 2007a,b; Yamada et al., 2006; Iughetti et al., 2007]. Although abnormalities of sleep, appetite, thirst, and fertility suggest hypothalamic dysfunction, radiologically evident abnormalities of the hypothalamus have not been described to date in PWS. Imbalances of neuropeptides have been noted but not generally replicated, and brain serotonin has not been directly

measured in individuals with PWS. Elevated levels of the serotonin metabolite 5-HIAA and neuropeptide Y were found in cerebrospinal fluid from a small sample of children with PWS compared to non-PWS children, although levels of 5-HIAA were comparable to matched obese children so this may not be a PWS-specific finding [Akefeldt and Mansson, 1998; Akefeldt et al., 1998, 2001]. Finally, a constellation of behavior problems that includes temper tantrums, obsessive-compulsive behavior, mood instability, skin picking, and maladaptive behavior typifies people with PWS and sets them apart from other intellectually disabled individuals. A dysfunction of the hypothalamic-pituitary axis likely accounts for relative growth hormone deficiency, poor appetite regulation, and sleep disorders. Other aspects are thought to have mixed origins: hypogonadotropic hypogonadism may have both hypothalamic and gonadal components [Brandau et al., 2008], hyperphagia stems from decreased satiation but also has components of obsessive-compulsive symptoms that may originate in the amygdala, thalamus, or orbitofrontal cortex. Maladaptive behavior and cognitive deficiencies suggest a disorder of the frontal lobe and associated structures.

Although the extrapolation of murine studies to human behavior must be approached with caution, studies of genetically engineered mice have successfully recapitulated the fundamental behavioral aspects of the respective human genetic disorder in many cases [Crawley, 2008]. In this case, general behavioral similarities between *Magel2*-null mice and common findings in PWS include anxiety-related traits particularly in new environments, a short-term memory deficit, and decreased voluntary activity; however, we did not identify abnormalities of learning in the mice that would parallel the intellectual disability that is essentially universal in PWS suggesting the contribution of a different gene(s) or a mouse-human difference in this respect.

Almost all individuals with PWS lack expression of multiple genes, including loss of function of *MAGEL2* and *neccin*, and most PWS candidate genes are moderately to highly expressed in the brain [Lee et al., 2003]. It is unclear how much contribution to the PWS phenotype is made by each of the deleted genes. Comparison with other mouse strains carrying individual PWS gene deletions may be informative, although few behavioral studies have been performed to date. We previously showed that mice lacking *neccin* are underweight at birth [Pagliardini et al., 2005], while another *neccin*-null strain has normal weight [Andrieu et al., 2006]. A preliminary behavior study of the latter strain of mice showed no behavioral changes in an open field test, but clear motor and sensory deficits secondary to neuronal defects in the developing nervous system [Andrieu et al., 2006]. Two mouse strains lacking the snoRNAs MBII-85 suffer from post-natal mild to severe growth retardation before weaning [Skryabin et al., 2007; Ding et al., 2008]. Behavior of MBII-85 mutant mice is also abnormal, with differences in tests of anxiety but not working or spatial memory, although a comparison group of similarly runted mice was not used in that study [Ding et al., 2008]. Thus, mice with individual deficiencies of three PWS candidate genes all have altered patterns of growth and behavior consistent with a multigenic origin for deficits in PWS.

A report of a child with atypical PWS carrying a chromosome deletion of 175 kb [Sahoo et al., 2008], and reports of individuals with PWS carrying translocations involving chromosome 15q11 that concurrently disrupt the activity of a set of small nucleolar

RNAs (HBII-85) (e.g., [Schule et al., 2005] and references therein), implicate the disruption of this region in the etiology of PWS while de-emphasizing the potential role of other genes. It is not possible to assess whether imprinting in the brain during pre-natal development of these rare individuals is affected by such sizeable genomic alterations within this complex imprinted region, thereby impinging on the normal expression of genes regulated by imprinting center transcript disrupted by these genomic events [Horsthemke and Buiting, 2006]. Firm evidence for the relative role of specific genes in abnormalities of brain and behavior in typical PWS therefore remains to be found. Our studies of this mouse model for *Magel2*-deficiency do however suggest a significant contribution of loss of *Magel2* to circadian dysfunction and predisposition to narcolepsy, increased adiposity and reduced muscle mass, learning and behavior, and infertility [Mercer and Wevrick, 2009], with a minor contribution to post-natal failure to thrive. Mouse models that examine the effect of ablation of individual PWS genes or these genes in combination will allow further dissection of this complex neurobehavioral condition.

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